INITIATION OF PROTEIN SYNTHESIS, III. FACTOR-GTP-CODON-DEPENDENT BINDING OF F-MET-TRNA TO RIBOSOMES

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Certain chemical information for the initiation of protein synthesis is contained in met-tRNA$_F$,[1] the formyl-accepting species of met-tRNA. Additional information, however, is conferred by enzymatic α-aminoacyl formylation of this compound. We have been interested in defining these two levels of chemical information and, in this regard, several points have become clear. Nonformylated met-tRNA$_F$ is selectively bound to ribosomes in appropriate phase when provided the AUG codon among an array.[2, 3] Formylation diminishes specific codon recognition by an AA-tRNA in nonenzymatic ribosomal binding and changes certain of its physical properties.[3] Further, certain factors, probably enzymatic, induce codon-dependent binding of F-met-tRNA$_F$ to ribosomes.[4, 5] Although AA-tRNA initiates protein synthesis *in vitro* at relatively high Mg^{2+} concentration,[6] under appropriate conditions, blocking of the α-amino group of AA-tRNA is *required* for formation of the initial peptide bond.[7]

In the studies described below, at least two and possibly three types of ribosomal binding reactions with varying specificities are discerned. The first is the codon-dependent binding of AA-tRNA.[8–10] The second type involves the binding of F-met-tRNA (but not its nonformylated analogue) to ribosomes in marked preference to other F-AA-tRNA's. This reaction depends upon the presence of codon, ribosomal factors, and GTP, and probably corresponds to that recently described by Lucas-Lenard and Lipmann[11] for the poly U, GTP, and factor-dependent binding of acetylphe-tRNA to ribosomes. It may also contribute to the GTP-dependent reaction observed by Hershey and Thach.[12] The third type, if it is distinct, is nonspecific, does not require added codon, but does require both GTP and soluble ribosomal factors. It may represent an extension of the first two. Each type of reaction is functional in that bound, formylated amino acids are transferred from tRNA to puromycin without further additions. The relation of each to a model for protein synthesis is considered.

Materials and Methods.—Preparation of ribosomes and ribosomal factors: Ribosomes washed in 1.0 *M* NH_4Cl were prepared from *E. coli* MRE-600, a low-nuclease strain isolated by Dr. H. E. Wade, by a method similar to that of Lucas-Lenard and Lipmann,[13] except that five 1.0 *M* NH_4Cl washes were carried out in 0.01 *M* $MgAc_2$. Ribosomes were stored at 4° in 1.0 *M* NH_4Cl; 0.01 *M* Tris-Cl, pH 7.2, 0.05 *M* KCl; 0.1 mM dithiothreitol; and 0.01 *M* $MgAc_2$. The ribosomal factors were isolated essentially as described by Stanley *et al.*[14] from two 1 *M* NH_4Cl ribosomal washings and precipitated in 70% $(NH_4)_2SO_4$. This material was used as such in these studies. It was composed of at least two factors which did not separate precisely as those described by Stanley *et al.*,[14] perhaps reflecting a strain difference.

Preparation and analysis of C^{14}-aminoacyl-, F-C^{14}-aminoacyl-tRNA, and trinucleotides: F-C^{14}-met and C^{14}-met-tRNA were prepared from *E. coli* B tRNA (used throughout) and characterized as described previously.[3] C^{14}-ile-tRNA and C^{14}-val-tRNA were formylated chemically using *p*-nitrophenyl-formate as described by Marcker and Sanger,[15] and characterized as was F-C^{14}-met-

tRNA. F-met-, met-, ile-, F-ile-, val-, and F-val-tRNA's were charged with 2.8, 2.1, 1.9, 1.7, 4.7, and 4.3 $\mu\mu$moles C^{14}-amino acid per 100 $\mu\mu$moles tRNA, respectively. Ninety-one per cent of the C^{14}-met in the unfractionated F-C^{14}-met-tRNA preparation was formylated, 85% of the F-C^{14}-val-tRNA, and 72% of the F-C^{14}-ile-tRNA. Control experiments using p-nitrophenyl-C^{14}-formate indicated that less than one nucleoside in 800 was formylated directly. Synthesis, purification, and characterization of the AUG, AUU, and GUU were carried out, with minor modifications, by use of the methods described by Leder, Singer, and Brimacombe.[16] Their purity was >98%.

C^{14}-AA-tRNA binding assay: The assay is that of Nirenberg and Leder.[17] Each 50-μl reaction mixture contained 0.1 *M* Tris-Ac, pH 7.2; 0.01 *M* $MgAc_2$ (or as otherwise noted); 0.05 *M* NH_4Cl; 1 A^{260} unit *E. coli* MRE-600 ribosomes; 0.15 A^{260} unit trinucleotides; approximately 6.0 $\mu\mu$moles C^{14}-AA-or F-C^{14}-AA-tRNA; 2 mM GTP (or as otherwise noted); and 5 μg ribosomal factors, where indicated. Incubation was at 23° for times indicated. Radioactivity of ribosome-bound C^{14}-AA-tRNA on nitrocellulose filters was determined in a liquid scintillation counter at an efficiency of 73%.[17] All determinations were made in duplicate.

Transfer assays: The assay for the formation of N-formyl-C^{14}-met-puromycin and C^{14}-met-puromycin is that of Leder and Bursztyn.[18] Contents of reaction mixtures and conditions of incubation were as noted in the binding assay. Where indicated, puromycin, 1 mM, was present in reaction mixtures. Following extraction with 1.5 ml ethyl acetate at pH 5.5 (for F-C^{14}-met-puro) or pH 8.0 (for C^{14}-met-puro), a 1.0-ml aliquot of ethyl acetate was removed and added to 10 ml of Bray's solution[19] for scintillation counting at 77% efficiency. Results are expressed as $\mu\mu$moles of compound contained in 1.0-ml aliquot counted.

Analysis of bound product: Duplicate binding reaction mixtures containing F-C^{14}-met-tRNA, GTP, factors, and AUG were incubated for 20 min, diluted to 10 ml with binding buffer, and centrifuged for 120 min at 100,000 $\times$ *g* in a Spinco no. 40 rotor. The supernatant was discarded, and the ribosomes were suspended in 0.1 ml NH_4OH 0.1 *N* and incubated at 37° for 60 min. Samples were then applied to Whatman no. 1 paper and subjected to high-voltage electrophoresis in 1.0 *M* pyridine acetate buffer, pH 3.6, at 70 volts/cm for 30 min. Authentic samples of F-met, met, met-phe, and F-met-phe were coelectrophoresed. Amino acids were detected by 0.25% ninhydrin stain; compounds with blocked α-amino groups were detected by the method of Bachur.[20] The radioactivity of 1-cm paper strips was determined in the liquid scintillation counter.[18]

Results.—Comparison of F-AA- and AA-tRNA in ribosomal binding and transfer reactions in the absence of factors and GTP: Using an unsupplemented binding system in which ribosomes are rate-limiting at 0.02 *M* Mg^{2+} (Table 1), C^{14}-met-, C^{14}-val-, and C^{14}-ile-tRNA are bound more efficiently to ribosomes in the presence of their appropriate codon than are their formylated analogues. Of the latter, F-met-tRNA is most efficiently bound and transferred to puromycin. In contrast to the less efficient binding response, significant transfer to puromycin occurs only in the case of the formylated AA-tRNA's, indicating a requirement for the blocking

TABLE 1

COMPARATIVE RIBOSOMAL BINDING AND TRANSFER TO PUROMYCIN OF F-C^{14}-AA- AND C^{14}-AA-TRNA

AA-tRNA	Trinucleotide Codon Plus ($\mu\mu$Moles Bound)	Trinucleotide Codon Minus ($\mu\mu$Moles Bound)	Puromycin Plus ($\mu\mu$Moles Transferred)	Puromycin Minus ($\mu\mu$Moles Transferred)
F-C^{14}-met-tRNA	1.43	0.37	1.17	0.06
C^{14}-met-tRNA	2.36	0.40	0.08	0.07
F-C^{14}-val-tRNA	0.57	0.36	0.36	0.15
C^{14}-val-tRNA	1.10	0.40	0.10	0.06
F-C^{14}-ile-tRNA	0.82	0.43	0.31	0.12
C^{14}-ile-tRNA	1.12	0.49	0.07	0.07

Reaction mixtures contained components and were incubated under conditions described under *Materials and Methods*. The appropriate codons used were: AUG, F-met- and met-tRNA; GUU, F-val- and val-tRNA; AUU, F-ile- and ile-tRNA. Incubation was for 20 min, without added GTP or factors.

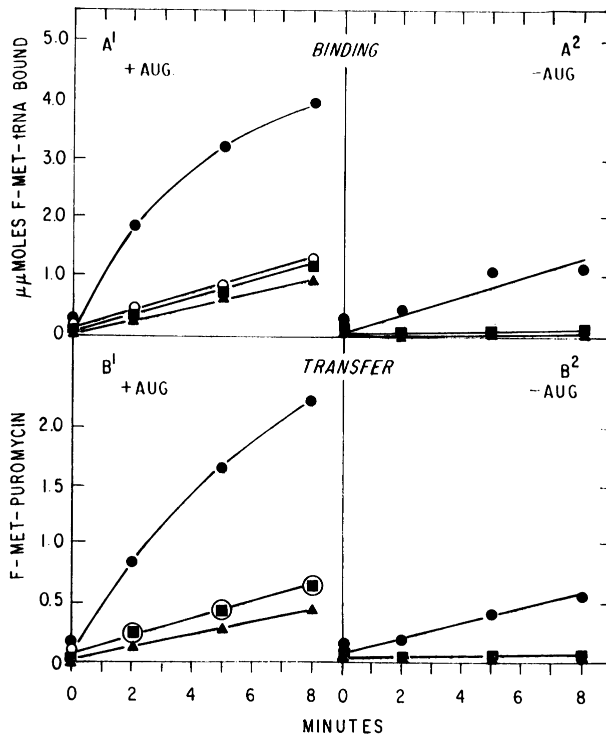

FIG. 1.—Time course of F-C^{14}-met-tRNA ribosomal binding and transfer reactions. Reaction mixtures contained components and were incubated under conditions described under *Materials and Methods* and as indicated in the figure. In the transfer reaction duplicate controls carried out in the absence of puromycin gave values for F-C^{14}-met-puromycin and C^{14}-met-puromycin of <0.03 μμmoles. Symbols represent the following: factors and GTP (●); factors (■); GTP, (▲); no addition (O).

group. There was little binding of val- or ile-tRNA at 0.01 *M* Mg^{2+} and practically no binding of the formylated compounds not shown. Consequently, there was no transfer of either to puromycin.[21]

Time course, requirements, and specificity of ribosomal binding and transfer reactions: Figure 1*A* shows the time course of binding of unfractionated F-C^{14}-met-tRNA to ribosomes at 0.01 *M* Mg^{2+}. A relatively slow reaction (0.2 μμmoles/min) independent of factors and GTP but requiring AUG and washed ribosomes can be noted. Another relatively slow reaction requiring ribosomal factors and GTP in the absence of codon can also be noted (0.2 μμmoles/min). A rapid reaction requiring factors, GTP, and AUG is also evident (1.0 μμmoles/min). In each case the kinetics of the transfer reaction (Fig. 1*B*) are identical, when corrected for size of aliquots assayed, to that of the binding reaction. GTP is required for the rapid, factor-dependent reaction. Figure 2*A* indicates the time course of the binding of nonformylated met-tRNA to ribosomes. This material consisted of >90 per cent formyl-accepting met-$tRNA_F$. Binding is rapid in the presence of AUG alone (1.2 μμmoles/min), but not appreciably influenced by GTP and/or ribosomal factors.

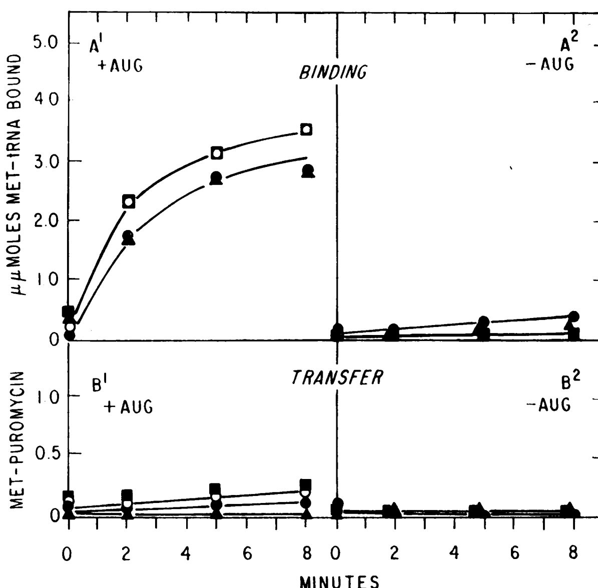

FIG. 2.—Time course of C^{14}-met-tRNA ribosomal binding and transfer reactions. Conditions and symbols as described under Fig. 1.

A slow GTP- factor-dependent binding reaction occurs in the absence of AUG (0.05 $\mu\mu$moles/min). Little transfer to puromycin (Fig 2B) occurs and that due to transfer of F-met contaminating the met-tRNA preparation (3%). In contrast to the slow codon-dependent, nonenzymatic binding of F-met-tRNA referred to in Figure 1, the rate and extent of factor-GTP-codon-dependent ribosomal binding of F-met- and of nonenzymatic met-tRNA binding are similar.

Characterization of the factor-GTP-codon-dependent ribosomal binding reaction of F-C^{14}-met-tRNA and its product: A concentration of GDP tenfold greater than that of GTP produces a 50 per cent reduction in the amount of F-C^{14}-met-tRNA bound to ribosomes in the presence of factors and AUG (Table 2). If GTP, factors, and ribosomes are pre-incubated in the absence of both F-C^{14}-met-tRNA and GDP, and subsequently in the presence of both, a similar inhibition is observed. This indicates that factor-GTP-codon-dependent binding involves a reaction that cannot occur in

TABLE 2

GDP INHIBITION OF FACTOR-GTP-DEPENDENT F-C^{14}-MET-tRNA BINDING TO PRE-INCUBATED RIBOSOMES

Addition	+AUG F-C^{14}-Met-tRNA ($\mu\mu$Moles bound)	-AUG
GTP 0.16 mM	1.15	0.17
GTP 0.16 mM + GDP 1.6 mM*	0.66	0.13
GTP 0.16 mM + GDP 1.6 mM	0.63	0.09
GTP 1.6 mM	1.83	0.47

* GDP added after 3-min pre-incubation.
Reaction mixtures contained components described under *Materials and Methods* and as indicated in the table. Mg^{2+} concentration was 0.02 *M* in this experiment only. Pre-incubation, prior to addition of F-C^{14}-met-tRNA, was for 3 min at 23°. F-C^{14}- met-tRNA was then added and incubation continued for 3 min at 23°. Five μg of ribosomal wash factors were added to each reaction mixture.

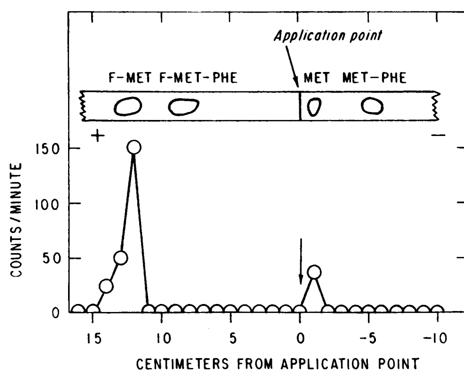

FIG. 3.—Electrophoretic analysis of the product of the factor-GTP-codon-dependent binding reaction. Details of hydrolysis and electrophoresis at pH 3.6 are given under *Materials and Methods.*

the absence of F-met-tRNA. The product of this reaction has been characterized by its electrophoretic mobility at pH 3.6 as shown in Figure 3. Ninety per cent of the counts are associated with the authentic F-met sample and 10 per cent with met.

Mg^{2+} dependence and tRNA species specificity of the factor-GTP-codon-dependent reaction: As seen in Figure 4, the factor-GTP-codon-dependent reaction readily occurs at Mg^{2+} concentrations as low as 0.005 M, whereas the codon-dependent and nonspecific interaction require higher Mg^{2+} concentrations for activity. This activity at low Mg^{2+} concentration has been observed by Lucas-Lenard and Lipmann[11]

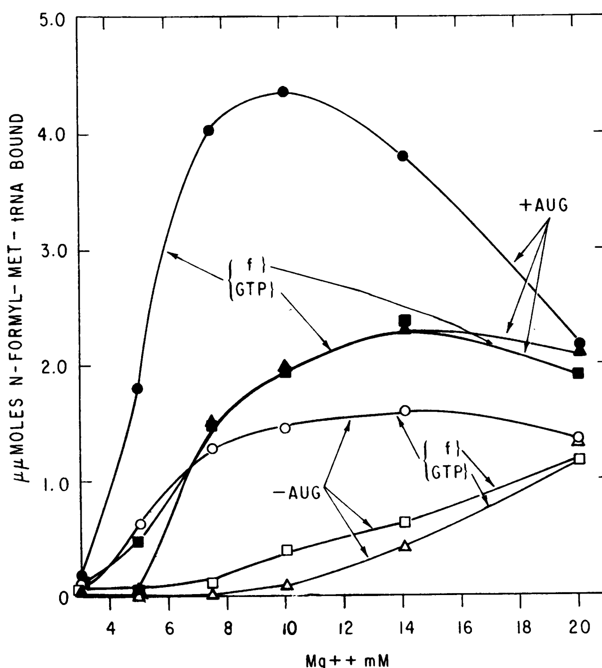

FIG. 4.—Mg^{2+} dependence of ribosomal binding reactions involving F-C^{14}-met-tRNA. Incubations were for 20 min. Symbols represent the following: factors, GTP and AUG (●); GTP and AUG (▲); factors and AUG (■); factors and GTP (O); GTP (△); factors (□).

TABLE 3

FACTOR-GTP-DEPENDENT BINDING OF F-C^{14}-AA- AND C^{14}-AA-TRNA'S TO RIBOSOMES AT 0.01 *M* Mg^{2+}

	Trinucleotide							
	AUG		AUG		AUU		GUU	
	+	−	+	−	+	−	+	−
	tRNA							
	F-C^{14}-met-		C^{14}-met-		F-C^{14}-ile-		F-C^{14}-val-	
Addition	($\mu\mu$Moles Bound)							
None	1.93	0.10	2.96	0.05	0.14	0.14	0.21	0.09
Factors, GTP	4.36	1.46	2.19	0.60	0.23	0.22	0.30	0.27

Reaction mixtures contained components and were incubated for 10 min under conditions indicated in *Materials and Methods* and in the table.

in the factor-GTP-dependent binding of acetylphe-tRNA to ribosomes in the presence of poly U.

The efficiencies (the amount bound compared to that added) of F-met-, met-, F-ile-, and F-val-tRNA's in the three ribosomal binding reactions can be compared in Table 3. As noted above, nonformylated met-tRNA's is bound more efficiently than F-met-tRNA, while F-ile- and F-val-tRNA's show little or nor response to their respective codons at 0.01 *M* Mg in the absence of factors and GTP. F-ile, F-val, F-phe, and F-glu are, however, bound to ribosomes at higher Mg^{2+} concentrations, but with less efficiency than their nonformylated analogues.[21] Factors and GTP stimulate binding of all four tRNA's, in the absence of added codons, but most efficiently the binding of F-met-tRNA. In experiments not shown, these components induce the binding of a number of formylated and nonformylated AA-tRNA's. The factor-GTP-codon-dependent reaction shows significant stimulation only in the case of F-met-tRNA.

Discussion.—The ribosomal initiation factors of Stanley *et al.*[14] have been shown by Lucas-Lenard and Lipmann[11] to require GTP for the poly U-directed binding of acetylphe-tRNA. In separate experiments Ravel[22] has isolated a supernatant factor from *E. coli* which, together with GTP and poly U, induces the binding of phe-tRNA to ribosomes in a reaction analogous to that observed in binding systems derived from reticulocytes by Schweet and co-workers.[23] Our experiments indicate that factor, GTP, and codon are required for the initial binding reaction. Similar observations have been made recently by Allende and Weissbach,[24] Levin,[25] and Wahba.[26] This binding is most efficient for F-met-tRNA, as compared with two other F-AA-tRNA's and nonformylated met-tRNA. Specificity has been suggested in studies by Salas *et al.*;[5] however, a GTP requirement was not apparent in their system. It should be emphasized that our studies and those of Salas *et al.*[5] were carried out in the presence of trinucleotide messengers.

A convenient model for protein synthesis[27] envisions an AA-tRNA binding site and a peptidyl-tRNA binding site on each ribosome. There is evidence for two binding sites per ribosome,[23, 28] though this has not been established unambiguously. The types of ribosomal binding described can be conveniently related to this model. The codon-dependent reaction may occur at the AA-tRNA site. Factor-GTP-codon-dependent binding might occur at the peptide site, possibly representing translocation of the tRNA from the AA-tRNA site at which the codon provided specificity. Direct access of F-met-tRNA to this site in the factor-GTP-codon-dependent reaction cannot be ruled out. The nonspecific factor-GTP-dependent binding may represent direct access of tRNA to the peptide site and may not involve interaction between tRNA and mRNA. This binding may correspond to the non-

specific binding observed by Cannon and de Groot and their coworkers[29, 30] or a less efficient variant of one of the codon-dependent types. It is unlikely that this reaction utilizes endogenous messenger, since no protein synthesis occurs in the absence of added mRNA and messenger activity would be detected in the nonenzymatic binding reaction.

It is difficult to apply usefully the criteria of puromycin transfer to define a ribosomal binding site.[31] As has been shown above, transfer to puromycin occurs following each of these binding reactions *only if* the α-amino group of the AA-tRNA is blocked. The blocking of this group may define access to the peptide binding site, but may also simply involve substrate specificity (as an analogue of peptidyl-tRNA) of the puromycin transfer activity apparently still associated with washed ribosomes. Recent evidence, in fact, suggests that under certain conditions the presumed anticodon-containing portion of F-met-tRNA is not required for puromycin transfer.[32] Thus the selective specificity of met-$tRNA_F$ may reside in its primary structure and define its uniquely efficient initial interaction with codon and ribosome. However, subsequent or concurrent enzymatic steps in initiation, e.g., enzymatic binding or translocation and initial peptide bond formation, appear to require the formylated derivative.

Summary.—At least two and possibly three types of tRNA binding to ribosomes can be distinguished in a system derived from *E. coli.* Each is functional in terms of undergoing subsequent peptide bond formation, but only when a formylated AA-tRNA is employed. The first and simplest system, well-described in the literature, requires codon and ribosome alone and has a high degree of amino acid-codon specificity. A second type of binding, requiring soluble ribosomal factors, GTP, and codon, has been demonstrated. In the presence of the appropriate trinucleotide codon, F-met-tRNA, but not F-ile- and F-val-tRNA, is induced to bind to ribosomes in this system. A third and possibly distinct type is nonspecific and occurs without added codon, but requires ribosomal factors and GTP.

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The following abbreviations are used: tRNA, transfer RNA; AA-tRNA, aminoacyl-tRNA; met-, F-met-, F-val-, F-ile-, and acetylphe-tRNA, methionyl-, formylmethionyl-, formylvalyl-, formylisoleucyl-, and acetylphenylalanine-tRNA, respectively; met-$tRNA_F$, formyl-accepting species of met-tRNA; A, adenosine; U, uridine; G, guanosine; poly U, polyuridylic acid; mRNA, messenger RNA; GTP, guanosine 5'-triphosphate. For oligonucleotides of specific structure, internal phosphodiesters (3'-5') between nucleosides are not indicated as in UU for diuridine monophosphate.

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